A fluorescent calmodulin that reports the binding of hydrophobic inhibitory ligands

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(Received 22 November 1982/Accepted 18 January 1983)

Ca^{2+} binding to calmodulin in the pCa range 5.5–7.0 exposes hydrophobic sites that bind hydrophobic inhibitory ligands, including calmodulin antagonists, some Ca^{2+} -antagonists and calmodulin-binding proteins. The binding of these hydrophobic ligands to calmodulin can be followed by the approx. 80% fluorescence increase they produce in dansylated (5-dimethylaminonaphthalene-1-sulphonylated) calmodulin (CDR_{DANS}). In the presence of Ca^{2+} , calmodulin binds the calmodulin inhibitor, R24571, with an affinity of approx. 2–3 nM and hydrophobic ligands, including trifluoperazine (TFP), W-7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide], fendiline, felodipine and prenylamine, with affinities in the micromolar range. This binding is strongly Ca^{2+} -dependent and Mg^{2+} -independent. Calmodulin shows a reasonably high degree of specificity in its binding of these ligands over other ligands tested. CDR_{DANS} , therefore, provides a convenient and simple means of monitoring the interaction of a variety of hydrophobic ligands with the Ca^{2+} -dependent regulatory protein, calmodulin. CDR_{DANS} binds to phospholipid vesicles made of (dimyristoyl)phosphatidylcholine (DMPC) or (dipalmitoyl)phosphatidylcholine (DPPC) and produces fluorescence increases only in the presence of Ca^{2+} and at temperatures above their gel-to-liquid crystalline phase transition. Although the fluorescence changes in CDR_{DANS} accurately report phase transitions in these liposomes, its binding to these vesicles is weak. Calmodulin probably requires a high-affinity lipid-bound receptor protein for its high-affinity binding to natural membranes.

Calmodulin is a 16 500-mol.wt. Ca^{2+} -binding protein that is considered to be ubiquitous in eukaryotic cells. It has been implicated as the Ca^{2+} receptor for a seemingly ever increasing number of Ca^{2+} -mediated events (Cheung, 1980; Means & Dedman, 1980; Klee *et al.*, 1980; Broström & Wolff, 1981). We and others have shown that Ca^{2+} binding to calmodulin results in large structural changes, including changes in its tyrosine fluorescence, near-u.v.-absorption spectrum and large increases in its α -helical content (Dedman *et al.*, 1977; Broström & Wolff, 1981). Tanaka & Hidaka (1980) and La Porte *et al.* (1980) have shown that Ca^{2+} -binding to calmodulin exposes hydrophobic

binding sites that are capable of binding a variety of hydrophobic fluorescent-probe molecules and calmodulin-binding proteins. Other drugs, including TFP and W-7, have been deemed calmodulin antagonists since they bind to calmodulin with reasonable affinity (K_d approx. $1 \mu\text{M}$) and inhibit calmodulin-mediated events with some specificity (Levin & Weiss, 1977, 1978; Hidaka *et al.*, 1979, 1980). Of particular interest is the new calmodulin antagonist drug, R24571, which is approx. 300 times more potent than TFP in inhibiting calmodulin-dependent phosphodiesterase (Van Belle, 1981*a,b*) and is a powerful inhibitor of the calmodulin-dependent Ca^{2+} -activated ATPase of red blood cells (Gietzen *et al.*, 1981).

Calmodulin-binding proteins, including myosin light-chain kinase, phosphodiesterase and troponin I, presumably bind to these same Ca^{2+} -induced hydrophobic domains on calmodulin and become activated (Tanaka & Hidaka, 1980; LaPorte *et al.*, 1980). For example, we (Johnson *et al.*, 1981) have

Abbreviations used: TFP, trifluoperazine; CDR_{DANS} , dansylated (5-dimethylaminonaphthalene-1-sulphonylated) calmodulin; DPPS, (dipalmitoyl)phosphatidylserine (dipalmitoylglycerophosphoserine); DPPC, (dipalmitoyl)phosphatidylcholine (dipalmitoylglycerophosphocholine); DMPC, (dimyristoyl)phosphatidylcholine (dimyristoylglycerophosphocholine).

shown that Ca^{2+} binding to calmodulin facilitates its binding to myosin light-chain kinase and the subsequent activation of this kinase, over the same pCa range where the hydrophobic sites on calmodulin are exposed. Thus, the Ca^{2+} -induced formation or exposure of these hydrophobic domains on calmodulin appear to be important for its interaction with the proteins it regulates and for the inhibition of calmodulin-mediated events, through a direct competition of hydrophobic ligands for these hydrophobic regulatory sites on calmodulin. The present paper represents our work with a dansylated calmodulin (CDR_{DANS}) that reports the interaction of hydrophobic ligands and calmodulin-binding proteins with calmodulin. By using this approach we have characterized the binding of a variety of Ca^{2+} antagonists and calmodulin antagonists to calmodulin.

Materials and methods

Calmodulin was purified from bovine testes by the method of Crouch *et al.* (1981). Calmodulin was dansylated by reacting 1 mg of protein/ml with a 5-fold molar excess of dansyl chloride (Pierce) in 10 mM-Mops (4-morpholinepropanesulphonic acid)/90 mM-KCl/2 mM-EGTA/3 mM- CaCl_2 , pH 7.0, for 24 h. This reaction mixture was then dialysed exhaustively against 10 mM-Mops/90 mM-KCl/2 mM-EGTA to remove excess free label. The bound dye concentration was determined by absorption spectroscopy using an absorption coefficient of $3980 \text{ M}^{-1} \text{ cm}^{-1}$ for the dansyl fluorophore (Johnson *et al.*, 1978) and the protein concentration was determined by the biuret reaction. Under these conditions 0.8–1.1 mol of the dansyl label was incorporated per mol of calmodulin. Liposomes were prepared by weighing 5–10 mg of lipid into a conical tube and dissolving each in chloroform. This lipid was dried under N_2 , layered with 2 ml of 10 mM-Tris/HCl/100 mM-KCl at pH 7.4 and then sonicated with a Heat Systems Microtip (model W-225R) sonicator for 10 min. This caused the suspensions to clear and these liposomes were centrifuged for 30 min at $160000g$ to remove any large particles. Phospholipid phosphorus concentrations were determined by the method of Bartlett (1959). Bovine brain DPPS and synthetic DPPC and DMPC were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Fluorescence studies were conducted on a Perkin-Elmer MPF-44A or 650-10S ratio-recording spectrofluorimeter operated in the ratio mode. Temperature was controlled by a Lauda K2R circulating-water bath and measured by a Bailey Instruments model Bat-4 probe thermometer.

The fluorescence-polarization studies with diphenylhexatriene were carried out on the MPF-44A

instrument with polarization accessory 063-0468, from Perkin-Elmer. The fluorescence polarization (P) was determined from the relation $P = (V_v - V_l)/(V_v + V_l)$, where V_v and V_l are the fluorescence intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam respectively. Polarization values were corrected for unequal dispersion of the parallel and perpendicular components of the light by the monochromators.

Results

The Ca^{2+} -induced exposure of hydrophobic binding domains in calmodulin

Upon binding to calmodulin the hydrophobic fluorescent probe molecule 8-anilino-naphthalene-1-sulphonate and the Ca^{2+} antagonist drug felodipine undergo a large increase in fluorescence (15-fold and 4.4-fold respectively), whereas the 'specific calmodulin inhibitor' TFP undergoes a small (20%) fluorescence decrease. These fluorescence changes can be used to monitor the Ca^{2+} dependence of the interaction of these hydrophobic ligands with calmodulin (Fig. 1). Between pCa 7.0 and pCa 5.5 (half-maximal near pCa 6.1) each of the hydrophobic ligands bind to calmodulin, and undergo their respective fluorescence changes. Mg^{2+} alone will not support ligand binding nor does 3 mM- Mg^{2+} affect the Ca^{2+} dependence of their binding. This would suggest that Ca^{2+} binding to Ca^{2+} -specific sites on calmodulin exposes hydrophobic domains on the protein to which these ligands bind.

Calmodulin antagonist and Ca^{2+} antagonist binding to calmodulin

Because the hydrophobic binding domains of calmodulin are so important in regulating the interactions of calmodulin with hydrophobic inhibitory ligands and with calmodulin-binding proteins, we wanted to develop a convenient means of monitoring calmodulin interactions with ligands and proteins. We have labelled calmodulin in 1:1 stoichiometry with dansyl chloride to form CDR_{DANS} . CDR_{DANS} binds 4 mol of Ca^{2+} /mol of protein and activates myosin light-chain kinase similarly to native unlabelled calmodulin (J. D. Johnson, T. H. Crouch & J. D. Potter, unpublished work). In the presence of Ca^{2+} , CDR_{DANS} undergoes a large (approx. 80%) fluorescence increase when Ca^{2+} antagonists or calmodulin antagonists bind to calmodulin. Fig. 2 shows the effect of various hydrophobic ligands on CDR_{DANS} fluorescence. The inset to Fig. 2 shows the 80% fluorescence increase and shift in λ_{max} from 515 to 497 nm, that occurs as prenylamine binds to CDR_{DANS} . Titrations of $2 \mu\text{M}$ - CDR_{DANS} at pCa 3.0 (or pCa 5.0) with calmodulin antagonists (R24571, felodipine, W-7 and

Fig. 1. The Ca^{2+} dependence of TFP, felodipine and 8-anilidonaphthalene-1-sulphonate binding to bovine testis calmodulin

The percentage of the total fluorescence increase in 8-anilidonaphthalene-1-sulphonate fluorescence (15-fold) (●) and felodipine fluorescence (4.4-fold) (▲) and the fluorescence decrease in TFP (20%) (○) are shown as a function of pCa. Each solution contained $10\text{ }\mu\text{M}$ -calmodulin and $50\text{ }\mu\text{M}$ ligand in 10 mM -Mops/ 90 mM -KCl/ 2 mM -EGTA/ 3 mM -MgCl₂, pH 7.0. They were titrated with Ca^{2+} as described previously (Johnson *et al.*, 1978). Excitation and emission pairs for 8-anilidonaphthalene-1-sulphonate, felodipine and TFP were 340/500nm, 365/445nm and 320/500nm respectively.

TFP) and with Ca^{2+} -antagonist drugs (fendiline, prenylamine, verapamil, D-600 and diltiazem) produced fluorescence increases (F/F_0) that were half-maximal ($M/2$) as indicated in Table 1. The apparent equilibrium constants (K_{app}) in Table 1 were determined by performing drug titrations at successively lower CDR_{DANS} concentrations ($1\text{ }\mu\text{M}$, $0.5\text{ }\mu\text{M}$, $0.2\text{ }\mu\text{M}$ and $0.1\text{ }\mu\text{M}$) and extrapolating the K_d to zero [calmodulin]. Clearly, the apparent affinities of the higher-affinity drugs (R24571, fendiline, prenylamine) were affected most markedly by lowering the calmodulin concentrations. Each of the Ca^{2+} antagonists gave relatively large (70–90%) enhancements except for W-7, which only produces a 20% enhancement. In the absence of added Ca^{2+}

Fig. 2. The effect of Ca^{2+} antagonists and calmodulin antagonists on CDR_{DANS} fluorescence

The percentage of the total fluorescence increase is shown as a function of added drug for R24571 (Δ), fendiline (○; Fen), prenylamine (●; PA), felodipine (■; Fel), W-7, (●), TFP (Δ), D-600 (□), verapamil (▲; Ver) and diltiazem (■; Dilt). The inset shows the fluorescence of CDR_{DANS} before (lower trace) and after (upper trace) the addition of $50\text{ }\mu\text{M}$ -prenylamine. Microlitre amounts of each drug, in aqueous solution, were added to CDR_{DANS} ($2\text{ }\mu\text{M}$) in 1 ml of 10 mM -Mops/ 90 mM -KCl/ 2 mM -EGTA/ 3 mM -CaCl₂ (pCa 3.0), pH 7.0 and the fluorescence intensity at 510nm was recorded. Excitation was at 340nm, except for TFP and felodipine titrations, where excitation was at 360nm and 290nm respectively, to prevent drug fluorescence. The fluorescence increase (F/F_0) produced by each drug is given in Table 1.

Table 1. The fluorescence enhancement (F/F_0) and half-maximal binding ($M/2$) in $2\text{ }\mu\text{M}$ - CDR_{DANS} with drug binding

The K_d values were determined by drug titrations at successively lower [CDR] and extrapolation of K_{app} versus [calmodulin] to zero [calmodulin].

Ligand	F/F_0	$M/2$ (μM)	K_d
R24571	1.90	1.5	2–3 nM
Fendiline	1.90	2.1	0.5–0.7 μM
Prenylamine	1.80	2.4	0.5–0.7 μM
Felodipine	1.93	5.5	2.8 μM
W-7	1.30	7.2	4 μM
TFP	1.60	9.5	5 μM
D-600	1.75	30	30 μM
Verapamil	1.74	30	30 μM
Diltiazem	1.76	80	80 μM

($\text{pCa} \geq 10$; 2 mM -EGTA), none of these hydrophobic ligands produced a fluorescence change in CDR_{DANS} . The presence of 3 mM -Mg²⁺ alone did not support the drug-induced fluorescence changes in CDR_{DANS} nor did it alter the drug dependence of the fluorescence increases they produce by binding to calmodulin in the presence of Ca^{2+} .

The ability of the calmodulin antagonists and Ca^{2+} antagonists to bind to CDR_{DANS} and produce a fluorescence increase seems to be somewhat specific for these compounds among the ligands tested. Lipids including DPPS (50 μM), DPPC (50 μM), palmitic acid (0.1 mM), oleic acid (0.1 mM) and cholesterol (10 μM), produced no fluorescence increases in CDR_{DANS} at 23°C. Atropine at 1 mM, isoprenaline, cyclic AMP, ATP, adenosine, tryptophan, histidine, ouabain, nitroprusside, nitroglycerine and papaverine at 0.1 mM produced no fluorescence changes in CDR_{DANS} . This suggests that these ligands do not bind to calmodulin in the concentration range tested. Among the phospholipids tested, DPPS does not bind, whereas DMPC was found to bind to CDR_{DANS} (above 23°C) and to produce an approx. 30% fluorescence increase (see below). In the presence of Ca^{2+} , calmodulin-binding proteins, including myosin light-chain kinase and troponin I, also produce large fluorescence changes in CDR_{DANS} , which allow us to follow their interaction with calmodulin (J. D. Johnson, unpublished work).

R24571 binding to CDR_{DANS}

We have found that the potent calmodulin antagonist, R24571, binds to CDR_{DANS} with much higher affinity than any of the other calmodulin inhibitors or Ca^{2+} antagonists tested. When 2 μM - CDR_{DANS} is treated with R24571, it undergoes an approx. 90% fluorescence increase, which is half-maximal near 1.5 μM -R24571 (Fig. 2) and an 18 nm blue shift (515 to 497 nm) in the wavelength of its emission maximum. Titrations of lower amounts of CDR_{DANS} with R24571 progressively increase the apparent affinity of R24571 for calmodulin. For example at 0.1 μM -calmodulin, the fluorescence increase is half-maximal at approx. 70 nM-R24571. Apparent equilibrium constants (K_{app}) were determined on titrations of 200, 150, 125, 100, 75, 50 and 25 nM-calmodulin and plotted as a function of calmodulin concentration. This gave a straight-line plot ($r = 0.998$) that intercepted the K_{d} axis (zero-concentration calmodulin) at 2.5 nM. This suggests that R24571 binds to CDR_{DANS} with an apparent affinity in the range of 2–3 nM. R24571 has, therefore, an affinity for calmodulin in the same range where it is able to inhibit calmodulin-activated brain phosphodiesterase and its binding to calmodulin may be easily monitored by CDR_{DANS} fluorescence.

Calmodulin binding to liposomes

Fig. 3 shows the fluorescence increase as CDR_{DANS} is titrated with DMPC lipid vesicles. In the presence of Ca^{2+} (pCa 3.0), and at 30°C, DMPC produces a 1.25-fold fluorescence increase. In the absence of Ca^{2+} at 30°C or in the presence of Ca^{2+}

Fig. 3. Effect of DMPC liposomes on CDR_{DANS} fluorescence

The fluorescence increase is shown as a function of added DMPC lipid in the presence of Ca^{2+} at 30°C (●) and 8°C (▲) and in the absence of Ca^{2+} at 30°C (○). DMPC lipid vesicles were added to 1 μM - CDR_{DANS} in 1 ml of 10 mM-Mops/90 mM-KCl/2 mM-EGTA, pH 7.0, with or without 3.0 mM- CaCl_2 and the fluorescence intensity was monitored at 510 nm.

at 8°C, DMPC does not produce a fluorescence increase in CDR_{DANS} . This suggests that both Ca^{2+} and temperature regulate the binding of DMPC to CDR_{DANS} . Fig. 4 shows the temperature dependence of this DMPC-induced fluorescence change in CDR_{DANS} . The fluorescence increase occurs with a midpoint near 23°C, the midpoint of the gel-to-liquid-crystalline phase transition of DMPC (Andrich & Vanderkooi, 1976). The temperature profile of this fluorescence increase is essentially identical with the temperature profile of DMPC phase transition as monitored by the diphenyl-hexatriene method (Fig. 4).

DPPC has a phase transition near 42°C. Titrations of CDR_{DANS} (pCa 3.0) with DPPC below 30°C produce no fluorescence increase, whereas a 1.25-fold increase is produced at 50°C. This does not represent a strong binding of CDR_{DANS} to either DMPC or DPPC lipid vesicles, however, since CDR_{DANS} is easily separated from DMPC lipid vesicles, even at 40°C (above phase transition) and in the presence of Ca^{2+} , by A15M chromatography (results not shown). It should be noted that lipid binding only produces a fraction (approx. 30%) of the fluorescence increase produced by drug binding. In the presence of lipid (and a 1.25-fold fluorescence increase), addition of drug (prenylamine or verapamil) produces an additional fluorescence increase, up to a 1.8-fold total increase, with

Fig. 4. The temperature dependence of the DMPC-induced fluorescence increase in CDR_{DANS}

The sample contained $1\mu\text{M}$ - CDR_{DANS} and $280\mu\text{g}$ of lipid/ml in 3 ml total volume of 10 mM-Mops/90 mM-KCl/2 mM-EGTA/3.0 mM- CaCl_2 , pH 7.0, held at 7.0°C . The reference solution was identical except that it contained no lipid. The fluorescence of the sample (F_s) minus the fluorescence of the reference (F_r) is plotted as a function of increasing temperature ($^\circ\text{C}$). The change in polarization of diphenylhexatriene in DMPC liposomes is shown ($\bullet$) as a function of temperature. The cuvette contained $200\mu\text{g}$ of DMPC phospholipid/ml at a diphenylhexatriene/phospholipid ratio of 1:200, in 10 mM-Tris/HCl/100 mM-KCl, pH 7.4. Excitation of diphenylhexatriene was at 335 nm and the emission was monitored through polarizers at 430 nm.

essentially the same midpoint as in the absence of lipid.

Discussion

Current evidence suggests that Ca^{2+} binds to calmodulin resulting in large structural re-arrangement, including the exposure or formation of hydrophobic domains on its surface. These hydrophobic domains are presumably the interfacial sites where calmodulin binds calmodulin-binding proteins, such as brain phosphodiesterase, myosin light-chain kinase, adenylate cyclase and various protein kinases (LaPorte *et al.*, 1980). Calmodulin binds (only in the presence of Ca^{2+}) and produces structural changes and activation of these proteins. Consistent with this, Johnson *et al.* (1981) have shown that the Ca^{2+} -calmodulin-induced structural changes in myosin light-chain kinase occur over the same Ca^{2+} concentration range that calmodulin binds to and activates myosin light-chain kinase (half-maximal near pCa 6.1).

A variety of inhibitory calmodulin antagonist drugs, TFP, R24571, W-7 and the fluorescence

hydrophobic probes 8-anilinoanthracene-1-sulphonate and 2-*p*-toluidinoanthracene-6-sulphonate have been shown to bind to these hydrophobic sites on calmodulin and to inhibit calmodulin activation of phosphodiesterase, myosin light-chain kinase and red cell Ca^{2+} -dependent ATPase. Presumably these hydrophobic ligands bind to some of the same hydrophobic sites on calmodulin as do the calmodulin-binding proteins and thereby act as competitive inhibitors of many calmodulin-calmodulin-binding protein interactions (Hidaka *et al.*, 1979; LaPorte *et al.*, 1980; Johnson *et al.*, 1982). TFP, 8-anilinoanthracene-1-sulphonate and the Ca^{2+} antagonist felodipine have all been shown to bind to calmodulin in the presence of Ca^{2+} (Levin & Weiss, 1977; LaPorte *et al.*, 1980; Boström *et al.*, 1981). We have used the intrinsic fluorescence of these ligands to monitor the Ca^{2+} dependence of their binding to calmodulin. We find that the Ca^{2+} -induced binding of all of these ligands to the protein is half-maximal near pCa 6.0–6.1. Under identical conditions, myosin light-chain kinase binding and activation by calmodulin is also half-maximal near pCa 6.1 (Johnson *et al.*, 1981) and phosphodiesterase activation by calmodulin is half-maximal near pCa 6.0 (Dedman *et al.*, 1977). Mg^{2+} alone will not support drug, MLCK or phosphodiesterase binding to calmodulin and 3 mM- Mg^{2+} has little effect on the Ca^{2+} dependence of their binding to calmodulin. This is consistent with the idea that Ca^{2+} binding to Ca^{2+} -specific sites on calmodulin exposes hydrophobic sites that serve as the interface for both calmodulin-calmodulin-binding protein interactions and calmodulin-hydrophobic-ligand interactions. This suggestion does not necessarily exclude other mechanisms of interaction of calmodulin with the proteins that it regulates. For example, calmodulin binds to a low-affinity (Ca^{2+} -dependent) and a high-affinity (Ca^{2+} -independent) binding site in phosphorylase *b* kinase. The Ca^{2+} -dependent low-affinity binding results in further activation of this kinase and may be through hydrophobic interactions, whereas the high-affinity binding may be of a quite different nature (Walsh *et al.*, 1980).

Our fluorescent calmodulin, CDR_{DANS} , serves as an accurate reporter of the binding of hydrophobic ligands to these sites on calmodulin. CDR_{DANS} undergoes an approx. 80% fluorescent increase with ligand binding. The binding constants of felodipine, TFP and W-7 with calmodulin, as determined by the fluorescence increase they produce in CDR_{DANS} ($K_d = 2.8\mu\text{M}$, $5\mu\text{M}$ and $4\mu\text{M}$ respectively), are very close to the K_d determined by other methods: calmodulin has been reported to have one to two binding sites for felodipine (K_d approx. $10\text{--}1\mu\text{M}$) (Boström *et al.*, 1981), two binding sites for TFP (K_d approx. $1\mu\text{M}$) (Levin & Weiss, 1977) and three binding sites for W-7 (K_d approx. $11\mu\text{M}$) (Hidaka

et al., 1980). R24571 has recently been introduced as a very potent inhibitor of calmodulin (Van Belle, 1981a,b). It can half-maximally inhibit calmodulin activation of brain phosphodiesterase at 5 nM. It must, therefore, have a very high affinity for calmodulin. We estimate its binding constant to CDR_{DANS} to be 2–3 nM. This is the highest affinity that has been reported for any ligand binding to calmodulin, suggesting that R24571 may be a quite specific calmodulin antagonist.

Some of the Ca²⁺ antagonists tested in the present study, namely fendiline and prenylamine, were not previously known to bind to calmodulin and yet we find that they bind with reasonably high affinity. In addition to their being Ca²⁺ antagonists (Fleckenstein, 1977) it should be recognized that they are also calmodulin antagonists and could alter many calmodulin-mediated events. Other Ca²⁺ antagonists, including verapamil, D-600 and diltiazem, bind to calmodulin but not with high enough affinity to be considered powerful calmodulin antagonists. The binding of these hydrophobic ligands exhibits some specificity and many hydrophobic ligands show no binding to CDR_{DANS} at all.

Phospholipid binding to calmodulin may involve a different site from the binding of calmodulin antagonists since they produce only a 25% fluorescence increase and they bind with a relatively low affinity. Calmodulin binds to liposomes only above their phase-transition temperature and the fluorescence increase in CDR_{DANS} with DMPC follows the same temperature profile as the gel-to-liquid-crystalline phase transition of the lipid. This suggests that phospholipids such as DMPC and DPPC can bind to calmodulin only after their hydrocarbon chains have melted. Furthermore, the fluorescence changes in CDR_{DANS} provide an accurate means of monitoring the phase transitions in these lipid vesicles. The association of calmodulin with these lipid vesicles is quite weak, since CDR_{DANS} does not co-chromatograph with the lipid even above its phase-transition temperature. This would suggest that for its high-affinity binding to biomembranes, calmodulin must interact with a membrane-bound protein and not lipid alone.

Calmodulin regulates a wide variety of Ca²⁺-dependent events in the cell. This is often accomplished by hydrophobic domains of calmodulin interacting with and activating various calmodulin-binding proteins. Hydrophobic ligands or drugs also interact with some of these hydrophobic domains on calmodulin and can in some cases compete with the binding of calmodulin-binding proteins. Drugs that can do this effectively have been called calmodulin antagonists (Kanamori *et al.*, 1981; Asano *et al.*, 1982). CDR_{DANS} provides a convenient means of monitoring both drug and protein interaction with calmodulin. Calmodulin interaction with cal-

modulin-binding proteins, including myosin light-chain kinase and troponin I (J. D. Johnson, unpublished work), can also be followed by the increases they produce in CDR_{DANS} fluorescence. CDR_{DANS} should, therefore, provide a useful tool for monitoring and characterizing the binding of calmodulin antagonists and many calmodulin-binding proteins to this important Ca²⁺-dependent regulatory protein.

Finally, when using CDR_{DANS} fluorescence to monitor drug binding to calmodulin, it should be remembered that the absorption and fluorescence spectra of the drug must be distinct from the absorption and fluorescence spectra of the dansyl group of CDR_{DANS}. For example, drugs that absorb strongly at 340 nm and fluoresce strongly at 510 nm cannot be easily tested for their binding to calmodulin by this method.

This work was supported by grants from the American Heart Association (79-1001) and the Muscular Dystrophy Association of America. We appreciate the excellent technical assistance of Mr. Douglas Fugman, the artwork of Ms. Gwen Kraft and the help of Ms. Joan Eichhold and Ms. Ginger Garrett in typing the manuscript. We gratefully acknowledge the gift of felodipine from Dr. Bengt Ljung of Hässle, Mölndal, Sweden, the gifts of diltiazem from Marion Laboratories, fendiline and prenylamine from Chinoin Pharmaceutical Co., TFP from Smith, Kline and French Laboratories and verapamil and D-600 from Knoll Pharmaceutical.

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